Exhibit B

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TECHNICAL ADVANCE

Construct design for efficient, effective and high-throughput gene silencing in plants

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Summary

Post-transcriptional silencing of plant genes using anti-sense or co-suppression constructs usually results in only a modest proportion of silenced individuals. Recent work has demonstrated the potential for constructs encoding self-complementary 'hairpin' RNA (hpRNA) to efficiently silence genes. In this study we examine design rules for efficient gene silencing, in terms of both the proportion of independent transgenic plants showing silencing, and the degree of silencing. Using hpRNA constructs containing sense/anti-sense arms ranging from 98 to 853 nt gave efficient silencing in a wide range of plant species, and inclusion of an intron in these constructs had a consistently enhancing effect. Intron-containing constructs (ihpRNA) generally gave 90–100% of independent transgenic plants showing silencing. The degree of silencing with these constructs was much greater than that obtained using either co-suppression or anti-sense constructs. We have made a generic vector, pHANNIBAL, that allows a simple, single PCR product from a gene of interest to be easily converted into a highly effective ihpRNA silencing construct. We have also created a high-throughput vector, pHELLSGATE, that should facilitate the cloning of gene libraries or large numbers of defined genes, such as those in EST collections, using an *in vitro* recombinase system. This system may facilitate the large-scale determination and discovery of plant gene functions in the same way as RNAi is being used to examine gene function in *Caenorhabditis elegans*.

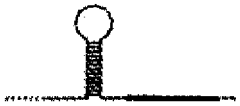
Keywords: PTGS, RNAi, genomics, vector, ihpRNA, Gateway.

Introduction

The ultimate goal of current genome projects is to identify the biological function of every gene in the genome. Whole genomes of several organisms (including *Arabidopsis*, http://www.arabidopsis.org), have been completely sequenced, providing a wealth of information. The functions of some of the genes have been identified directly by the appropriate assay, or have been inferred by homology to genes of known function in other organisms. Loss-of-function mutants, from insertional mutagenesis or transposable elements, have also been very informative about the role of some of these genes (AzpirozLeehan and Feldmann, 1997; Martienssen, 1998), particularly in the large-scale analysis of the yeast genome (Ross-Macdonald *et al.*, 1999). However, the functions of a large proportion of genes remain unknown.

Injection or ingestion of dsRNA into nematodes can trigger specific RNA degradation, in a process known as RNA-interference (RNAi; Fire *et al.*, 1998). This process facilitates targeted post-transcriptional gene silencing (PTGS) and has recently been harnessed to study the function of over 4000 genes on chromosomes I and III

(a)

Construct	Predicted RNA structure	PTGS	*n*
Adj-hp RNA		25%	8
hpRNA		85%	14
sRNA		30%	10

(b)

hpRNA		55%	234
ihpRNA		90%	243
ihpRNA overhang		80%	48
ihpRNA spacer		89%	36

Figure 1. The predicted RNA structure and efficacy of gene-silencing constructs.
(a) Three constructs, controlled by Ubi1 promoter, silencing GUS in rice. Thick lines indicate a 560 nt GUS sequence; grey lines indicate non-GUS sequences; dashed grey lines indicate intron-junction sequences left after splicing; and short lines within the stem of hairpin structures indicate base pairing. Numbers in PTGS column indicate the percentage of plants showing GUS silencing; *n* = number of plants in each treatment.
(b) Silencing efficacy of four different construct types with sequences as depicted in (a), except the thick lines in hpRNA and ihpRNA represent the various different target sequences in Table 1; and the thick lines in iphRNAoverhang and iphRNAspacer represent PVY Pro sequences. The percentage PTGS of hpRNA and ihpRNA, and iphRNAoverhang and iphRNA spacer, are the average percentage silencing of these types of constructs reported in Table 1 and the percentage of plants showing immunity to PVY, respectively.

in *Caenorhabditis elegans* (Fraser *et al.*, 2000; Gonczy *et al.*, 2000). We discovered that transgenes designed to express double-stranded or single-stranded self-complementary (hairpin) RNA have a similar post-transcriptional silencing effect in plants (Wang and Waterhouse, 2000; Waterhouse *et al.*, 1998) and that, in at least two examples, almost 100% of plants transformed with an intron-containing hairpin RNA construct showed silencing (Smith *et al.*, 2000). These results led us to ask whether hpRNA technology might be exploited for gene discovery in plants. We have sought to design and evaluate generic intron–hpRNA constructs that might enable plant gene-discovery studies on a scale that matches those in nematodes.

Results

Location of silence-inducing sequences in hairpin RNA constructs

Constructs encoding RNAs with regions of self-complementarity efficiently induce gene silencing. We have previously shown that the sequences in the duplex stem in hpRNAs direct gene silencing (Smith *et al.*, 2000; Waterhouse *et al.*, 1998), whereas the results of Hamilton *et al.* (1998) suggest that single-stranded RNA sequences adjacent to a potential hairpin-forming structure give sequence specificity to silencing. The latter arrangement (adj-hpRNA) could be easily incorporated into gene-silencing vectors as the sequence encoding the hairpin RNA could be generic to the vector, while the specificity of the silencing would be accomplished by simply inserting a single copy of target gene sequence. In contrast, hpRNA constructs require two copies of the target sequence in an inverted-repeat orientation, in order to produce duplex RNA. To compare the relative efficacy of the designs, various GUS-silencing constructs, under the control of the Ubi1 promoter and associated intron, were made (Figure 1a) and super-transformed into GUS-expressing rice. Histochemical staining of the transformed plants showed that the adj-hp RNA construct gave no higher frequency of silenced lines than conventional co-suppression (sRNA), but the hpRNA construct gave many more silenced lines (Figure 1a). This suggested that the hpRNA was the design of choice.

Examination of the stained rice Ubi-hpGUS plants and a similar 35S-hpGUS construct in tobacco (Figure 2f) showed that the silencing was evenly distributed throughout the plant. Analysis of RNAs in the tobacco plants for the presence of GUS-derived small (≈21 nt) RNAs showed a perfect correlation between the presence of these molecules and the presence of the 35S-hpGUS construct and silencing of the target GUS gene (Figure 3). This confirms that the silencing was due to PTGS; such small RNAs are a hallmark of PTGS (Hamilton and Baulcombe, 1999; Waterhouse *et al.*, 2001)

Intron-spliced hpRNA vectors

We have found that it is necessary to include a spacer region between the arms of hpRNA constructs for stability of the inverted repeat DNA in *Escherichia coli*. However, replacing the spacer (loop) region of hpRNA constructs with a functional intron sequence increases the proportion of independent silenced lines recovered from approximately 50 to about 100% (Smith *et al.*, 2000). In these experiments, the targets were potato virus Y (PVY) and the *FAD2* Δ12-desaturase gene of *Arabidopsis*. The constructs were designed such that their pre-mRNA should splice to

form hpRNAs with small loops (Figure 4). The PVY construct should give an hpRNA comprising a 730 nt stem and a 6 nt loop; the hpRNA from the Δ12-desaturase construct should contain a 120 nt stem and a 21 nt loop. Although the PVY construct contained only two and four bases of original exon sequence 5′ and 3′ of the intron, respectively, the intron was still functional. RT–PCR and sequencing of transgene mRNA in plants containing the *Hin*dIII intron fragment (Figure 4) showed that the intron was cleaved out, leaving the predicted splice junction (data not shown).

The Δ12-desaturase result (100% of independent plants showing silencing despite having a 21 nt loop in the hpRNA) showed that the intron-enhanced silencing was not solely due to the tightness of the hairpin loop. Therefore we wondered whether this could be exploited to make a generic intron-spliced hpRNA (ihpRNA) vector into which the gene, or gene fragment, of choice could be directionally cloned to make sense and anti-sense arms. The vector pHANNIBAL (Figures 4 and 5), and a sister vector, pKANNIBAL (with bacterial ampicillin and kanamycin resistance genes, respectively), were designed so that a PCR fragment could be inserted in the sense orientation into the *Xho*I.*Eco*RI.*Kpn*I polylinker and in the anti-sense orientation in the *Cla*I.*Hin*dIII.*Bam*HI.*Xba*I polylinker. This may be accomplished either by two separate PCR reactions with the appropriate single sites introduced with each primer, or by a single PCR using primers each introducing two restriction sites (e.g. primer 1, *Xba*I.*Xho*I.xxx; primer 2, *Cla*I.*Kpn*I.xxx). The construct will produce an hpRNA with a loop of 30–50 bases depending on which restriction sites are used to insert the targeting gene sequences.

The efficacy of pHANNIBAL was tested in *Arabidopsis* targeting the pigment biosynthesis gene chalcone synthase (*CHS*); the ethylene signalling gene *EIN2*; and the flowering repression gene *FLC1*. These genes were chosen because their mutant alleles have been reported in *Arabidopsis* to give distinct phenotypes. The *tt4* (CS85) EMS mutant (Koornneef *et al.*, 1990) produces inactive CHS, resulting in reduced production of flavonoid pigments in both the stem and seed coat. The mutant *ein2* (Alonso *et al.*, 1999) is insensitive to ethylene and grows well on media containing 1-aminocyclopropane-1-carboxylic acid, whereas wild-type plants develop a very stunted appearance when grown on such media. The mutant *flc1* (Amasino *et al.*, 2000) flowers earlier than wild-type *Arabidopsis*.

A 741 nt piece of CHS coding region was amplified from *A. thaliana* (Landsberg *erecta*) using primers that added an *Xho*I and a *Kpn*I site on the ends of one product and an *Xba*I and *Bam*HI site on the ends of the other product. These two amplification products were then directionally cloned into pHANNIBAL (Figure 5). Similar cloning strategies were adopted for a 600 nt sequence from *EIN2*, and both a 650 nt and a 400 nt sequence from *FLC1*. As controls, sense and anti-sense constructs of CHS and an anti-sense construct of FLC1 were also generated. All the constructs were subcloned into the binary vector pART27 and transformed into *Arabidopsis*.

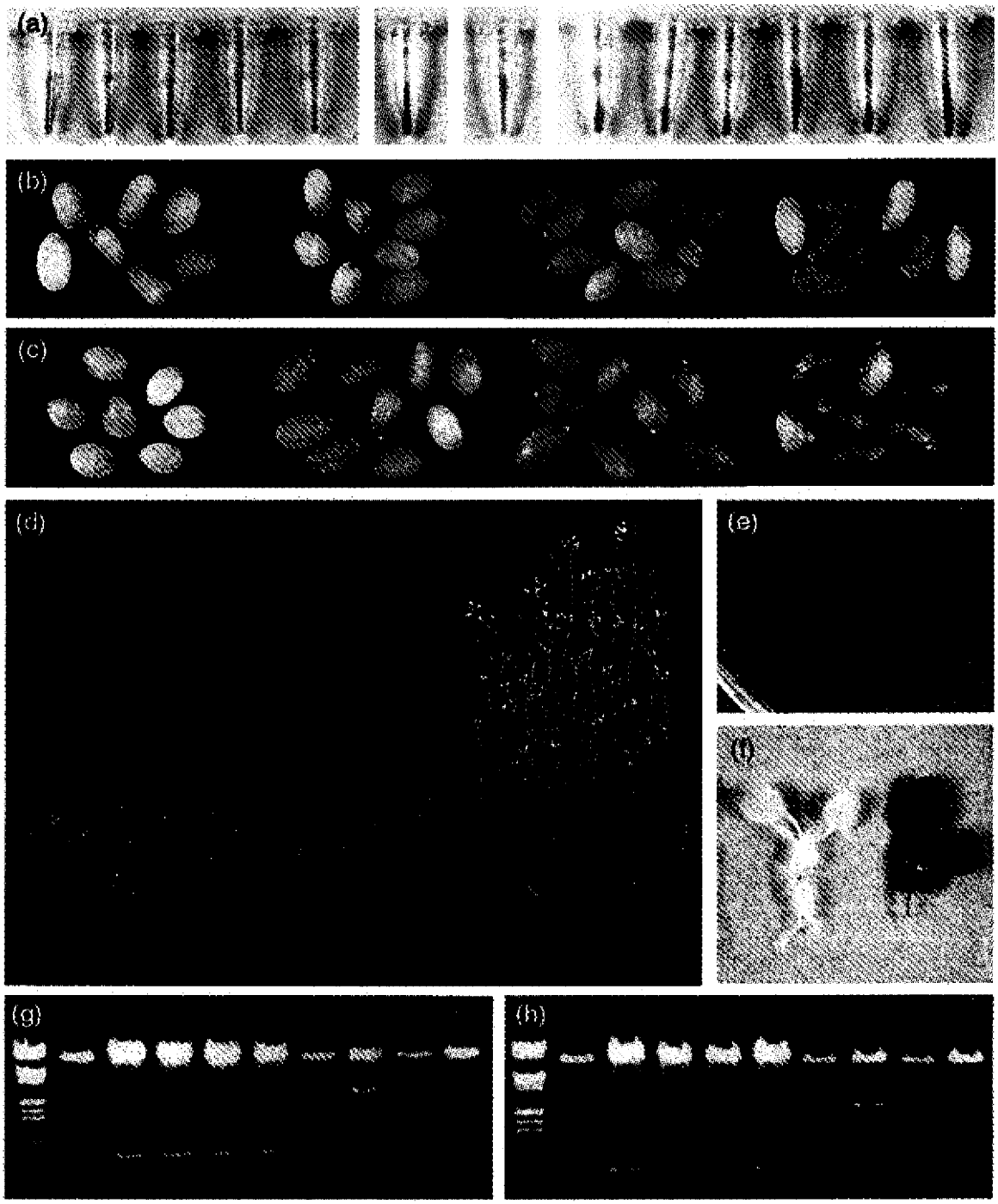
Only two of the 19 plants transformed with the CHS co-suppression construct, and none of the 25 plants transformed with the CHS anti-sense construct, showed any obvious evidence of silencing. Whereas over 90% (21 of 23) of the plants transformed with the CHS-HANNIBAL constructs showed pronounced silencing (Table 1). The seed colours of most of these lines were virtually indistinguishable, to the naked eye, from seed of the *tt4*(CS85) mutant (Figure 2a). Examination of the seed under a light microscope revealed that the degree of pigmentation was generally uniform in the cells of the coat of an individual seed, and among seeds of the same line (Figure 2b,c). There was a perceptible difference in the levels of pigmentation between the different lines. The relative flavonoid content of seed from three lines selected to span the range of seed colour in the plant lines transformed with CHS-HANNIBAL, and from the co-suppression line giving the lightest coloured seed were 7, 23, 47 and 75%, respectively. The *tt4* (CS85) and wild-type seed had values of 0 and 100%, respectively.

Sixty-four independent lines transformed with the EIN-HANNIBAL construct were obtained. The progeny from 42 of the lines showed Mendelian segregation for normal and stunted growth when grown on ACC medium, whereas all wild-type plants showed a stunted morphology on this medium (Table 1; Figure 2e).

The transgenic progeny of every one of the 31 independent plants transformed with the FLC1-HANNIBAL constructs flowered earlier and made fewer leaves, prior to flowering, than wild-type plants (Table 1; Figures 2d and 6). The transgenic progeny from the majority of the plants transformed with the FLC1 anti-sense construct had similar leaf numbers and flowering times to those of wild-type plants. The flowering time and leaf number provided an easy measurement of the degree of silencing in individual lines. Only two of the 31 FLC1-HANNIBAL plant lines did not flower in less than 25 days after germination, whereas only two anti-sense plant lines flowered in less than 30 days. This suggests that ihpRNA constructs not only give an increased proportion of silenced transformants than anti-sense constructs, but also give more profound levels of silencing. However, even the most profoundly silenced FLC1-HANNIBAL line flowered 1 day later than the *flc* mutant, suggesting that it was not quite the equivalent of a null allele.

Collectively, pHANNIBAL-based constructs (which are driven by the constitutive 35S promoter) have been

made for five different genes (*CHS, EIN2, FLC1*, PVY-Pro and polyphenol oxidase – *PPO*). Similar intron-containing constructs targeting seed specifically against two different genes (Δ12- and Δ9-desaturase) in *Arabidopsis* and/or cotton have been tested for their silencing efficiency. Intron-less hpRNA, anti-sense and co-suppression constructs have also been used in many of these gene/host combinations. The results are summarized in Table 1. The ihpRNA constructs were effective, with arm lengths ranging from 98 to 853 nt, giving 66–100% (average 90%) independent silenced transformants. Intron-free hpRNA constructs gave 48–69% (average 58%) silenced



transformants, and conventional co-suppression or anti-sense constructs gave 0–30% (average 13 and 12%, respectively) silenced transformants. The intron-spliced or intron-free hpRNA constructs were effective when targeted against the coding, 5′ untranslated or 3′ untranslated regions of the mRNA. Taken together, these results indicate that ihpRNA constructs consistently give the most efficient silencing under a wide range of conditions.

Effect of intron location and unbalanced arms in pHANNIBAL

Intron-spliced hpRNA constructs appear to give a higher proportion of silenced transformants than intron-free hpRNA constructs. One explanation for this might be that the process of intron-splicing aligns the arms of the hpRNA, facilitating their duplex formation in the spliceosome complex, whereas the arms of hpRNAs have to find their self-complementarity by random, but tethered, collisions. If there is a threshold of duplex RNA required for PTGS in plants, then facilitating more efficient duplex RNA formation from ihpRNA might raise the level in low transgene-expressing plants such that PTGS is enabled. Similar levels of transcription of non-spliced hpRNA might produce lower steady-state levels of duplex RNA that are insufficient for PTGS. The same threshold theory could also be applied for the tighter loop of ihpRNA, giving more nuclease-stable and higher steady-state duplex RNA levels than the larger looped hpRNA. To test the validity of these possibilities, a construct was made in which a spacer region was inserted between one of the arms and the intron in a PVY ihpRNA construct (Figure 1b). This spacer region should impede alignment of the arms during the splicing process and produce a spliced hpRNA with a large loop. When plants transformed with the construct were challenged with PVY, 32 out of 36 independent transformants were immune to the virus. This suggests that the majority of the intron-enhanced silencing efficiency is not due to better alignment of the RNA arms or by presence of a tighter ssRNA loop. It may also explain why the GUS–hpRNA construct so efficiently silenced GUS in the rice plants reported in Figure 1(a), as this construct contained an intron in the 5′ untranslated leader sequence of the ubiquitin promoter.

A common feature of our hpRNA constructs has been the use of matched-length arms. These constructs should produce hpRNA with only a few unpaired 5′ nucleotides. If pHANNIBAL is to be used as a generic vector for inserts from gene libraries, occasionally a restriction site within the PCR fragment will be common to the one used to clone into the polylinker. This will sometimes lead to the unintended construction of an hpRNA with unmatched arm length. To investigate whether this was an important attribute, a pHANNIBAL construct was made (using the

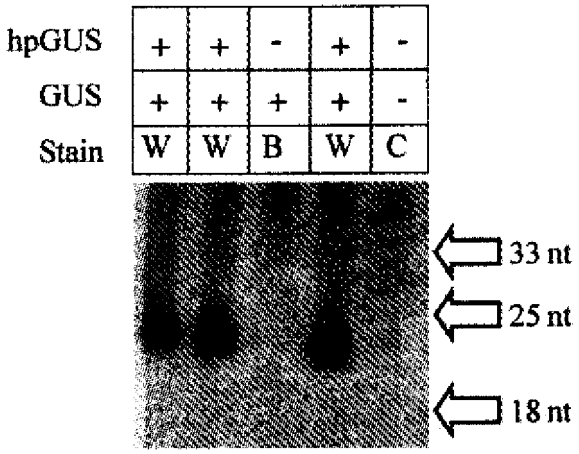


Figure 3. Detection of short (≈22 nt) GUS-derived RNAs in tobacco plants showing hpRNA-mediated GUS silencing.
Total RNA (20 μg each), isolated from tobacco plants showing independent segregation of a 35S GUS transgene and a 35S hpGUS transgene, was separated in a 15% denaturing polyacrylamide gel, blotted onto a Hybond-N membrane and hybridized with *in vitro*-transcribed ^{32}P-labelled GUS RNA. The presence (+) or absence (–) of the target GUS and/or the 35S hpGUS transgene in the plant, from which the sample was taken, is indicated above each track. The phenotype of each of these plants after incubation with X-glucuronide and removal of chlorophyll with ethanol is also indicated. Silenced lines are white (W), unsilenced lines blue (B). The non-transgenic control is designated (C). Sizes indicated on the filter were determined by migration of DNA oligonucleotides.

Figure 2. Silenced phenotypes in ihpRNA transformed plants and recombinase cloning into pHELLSGATE.
(a) *Arabidopsis* seed samples from left to right: five independent CHS-ihpRNA lines; wild-type seed; *tt4*CHS mutant; three CHS-anti-sense lines and three CHS-co-suppression lines. The anti-sense and co-suppression lines were chosen as those showing the lightest seed-coat pigmentation.
(b) *Arabidopsis* seed samples from four independent CHS-ihpRNA lines, chosen to reflect the range of seed-coat pigmentation, viewed under a light microscope.
(c) Four companion seed samples to (b) from left to right: *tt4*CHS mutant; the two anti-sense-silenced lines from Table 1; and wild-type seed.
(d) Three pots of *Arabidopsis* plant lines 25 days after germination. From left to right: wild-type; earliest-flowering anti-sense; and FLC1-pHANNIBAL-transformed line.
(e) *Arabidopsis* transformed with EIN2-pHANNIBAL growing on 50 μM ACC. The larger, vigorous plantlet is an ethylene-insensitive EIN2-pHANNIBAL plant; the small plantlets are ethylene-sensitive wild-type plants.
(f) X-glucuronide-stained transgenic GUS tobacco plantlets segregating for presence (left) or absence (right) of the GUS-hpRNA transgene.
(g,h) Agarose gel of restriction enzyme-digested plasmid preparations from nine individual colonies recovered from *E. coli* transformed with a pHELLSGATE/400ntPCR-product recombination reaction. *Xho*I digestion (g) will release the 400 nt sense arm but not the ccdB fragment, and *Xba*I digestion (h) will release the 400 nt anti-sense arm but not the other ccdB fragment. Left-hand track in both gels contains size markers.

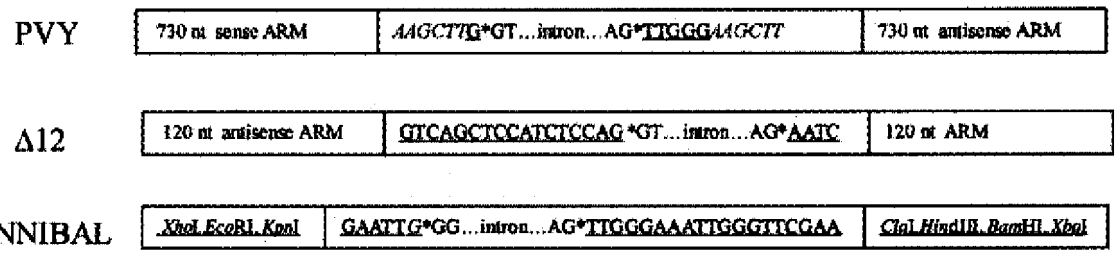


Figure 4. Splice junctions, loop regions and self-complementarity of ihpRNA constructs: PVY, Δ12-desaturase, and pHANNIBAL.
Nucleotides underlined have no complement in the hpRNA and should form a loop structure. *, splice point; *Hin*dIII site in PVY-ihpRNA construct shown in italics.

PVY–Pro sequence) that should produce a hpRNA with a stem of 400 nt and 5′ region of 300 unpaired nucleotides (Figure 1b). When 48 independent transformed plants, containing this construct, were challenged with PVY, 38 (≈80%) of them were immune to the virus. This shows that an unpaired 5′ extension of hpRNA does not abolish its ability to induce silencing, although its efficiency may be slightly reduced.

High-throughput vector

With the completion of the *Arabidopsis* genome project; the advent of micro-array technology; and the ever-increasing investigation into plant metabolic, perception and response pathways, a rapid, targeted way of silencing genes would be of major assistance. The high incidence and degree of silencing in plants transformed with pHANNIBAL constructs suggest that it could form the basis of a high-throughput silencing vector. However, one of the major obstacles in using pHANNIBAL for a large number of defined genes or a library of undefined genes would be cloning the hairpin arm sequences for each gene in the correct orientations.

Attempts to clone PCR products of sense and anti-sense arms, together with the appropriately cut pHANNIBAL vector as a single-step four-fragment ligation, failed to give efficient or reproducible results (data not shown). Therefore a construct (pHELLSGATE) was made (Figure 5) to take advantage of Gateway technology which facilitates easy cloning of PCR fragments (http://www.invitrogen.com/content.cfm). With this technology, a PCR fragment is generated (bordered with recognition sites attB1 and attB2) which is directionally recombined *in vitro* into a plasmid containing attP1 and attP2 sites using the commercially available recombinase preparation.

The pHELLSGATE vector was designed such that a single PCR product from primers with the appropriate attB1 and attB2 sites would be recombined into it simultaneously to form the two arms of the hairpin (Figure 5). The *ccdB* gene, which is lethal in standard *E. coli* strains such as DH5α (but not in DB3.1), was placed in the locations to be replaced by the arm sequences, ensuring that only recombinants containing both arms would be recovered. Placing a chloramphenicol-resistance gene within the intron gives a selection to ensure the retention of the intron in the recombinant plasmid. The pHELLSGATE vector was tested using 200 and 400 nt PCR products for two different genes. Many bacterial colonies were obtained on chloramphenicol-containing plates spread with DH5α bacteria, transformed with the *in vitro* recombination reaction. Analysis of 24 colonies transformed with the 400 nt reaction and 36 colonies from the 200 nt reaction showed that, in both cases, all but one of the colonies contained the desired recombinant plasmid (Figure 2g,h). This was confirmed by sequence analysis (data not shown). These results show that this vector facilitates the rapid, efficient and simple production of hpRNA constructs. pHELLSGATE is a binary vector, with a high-copy-number origin of replication for ease of handling. Recombinant pHELLSGATE constructs can be directly transformed into *Agrobacterium* for transformation into plants. This system should lend itself to high-throughput applications.

Discussion

Now that the genomes of a number of species have been completely sequenced, the challenge is to understand the functions and interplay of genes in an organism. The use of chemical mutagens, transposons and T-DNA tagging have been very useful in screening for mutants of individual genes. However, with these undirected methods it is often slow and laborious work to identify each mutant and to track down the gene responsible. RNAi has revolutionized the study of genes in *C. elegans* and *Drosophila*, with two groups recently reporting the systematic analysis of over 4000 genes on chromosomes I and III in *C. elegans* (Fraser *et al.*, 2000; Gonczy *et al.*, 2000). By way of comparison, chromosome 2 of *Arabidopsis* has been entirely sequenced (Lin *et al.*, 2000) and the presence of 4037 genes has been predicted. Yet to undertake a systematic analysis

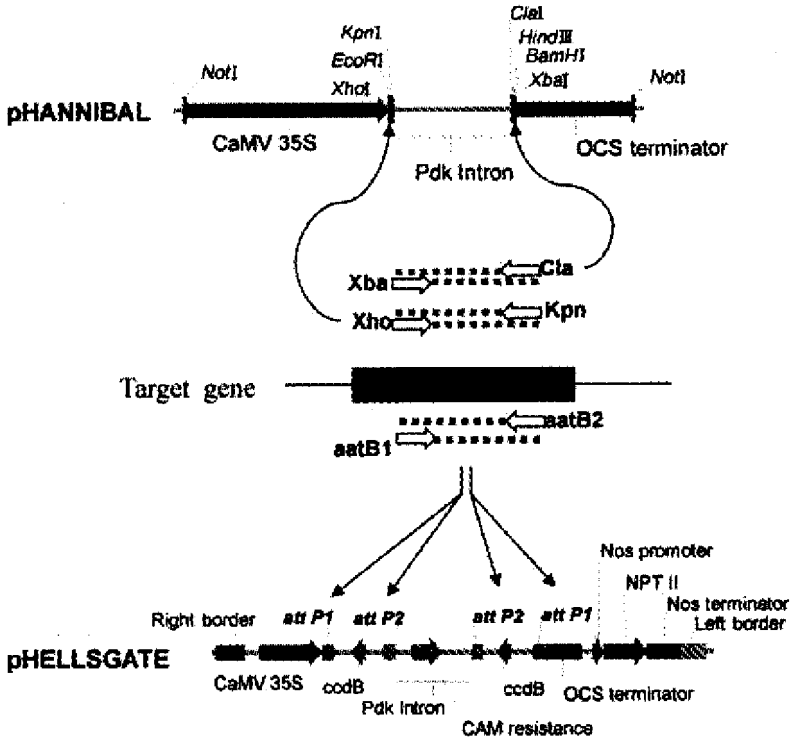


Figure 5. Maps and cloning strategies for pHANNIBAL and pHELLSGATE.
PCR products from the target gene are cloned into the polylinkers of pHANNIBAL conventionally; restriction sites added by the primers ensure the correct orientation of the resulting sense and anti-sense arms. The attB1 and attB2 sequences on a single PCR product facilitate the recombination of one sense-orientated and one anti-sense-orientated molecule into each molecule of pHELLSGATE when incubated with BP clonase. The complete sequences and annotations for pHANNIBAL and pHELLSGATE have been lodged at EMBL (Acc No: AJ311872 and AJ311874).

Table 1. Efficiency of hpRNA, co-suppression and anti-sense constructs at silencing a range of genes in a range of plant species

Gene	Species	Prom	Intron	Target	Arm (nt)	GenBank Acc No.	Co-ordinates	ihp RNA	hp RNA	Sense	Anti-sense
PPO	Tobacco	35S	Pdk	ORF	572	AX028815	172–844	21/30		5/54	
GUS	Tobacco	35S		ORF	800	S69414	1–800		23/48		
PVY	Tobacco	35S	Pdk	ORF	730	U09509	6278–7008	23/24	25/43	2/27	1/25
EIN2*	*Arabidopsis*	35S	Pdk	ORF	600	AF141202	538–1123	42/64			
FLC1*	*Arabidopsis*	35S	Pdk	ORF	650	AY034083	1–650	16/16			2/13
FLC1*	*Arabidopsis*	35S	Pdk	ORF	400	AY034083	250–650	15/15			
CHS	*Arabidopsis*	35S	Pdk	ORF	741	AF112086	248–1075	21/23		2/19	0/25
Δ12	*Arabidopsis*	Napin	Δ12a	3′ UTR	120	L26296	1243–1363	30/30	44/63	4/41	3/21
Δ12	Cotton	Lectin		ORF	853	X97016	68–921		17/29		7/30
Δ12	Cotton	Δ12c	Δ12c	5′ UTR	98	X97016	1–98	26/26			
Δ9	Cotton	Lectin		ORF	514	X95988	24–538		15/26		4/30
GUS	Rice	Ubi		ORF	560	S69414	1–560	12/14		3/10	1/8
Average percentage of silenced plants								90	58	13	12

The type of promoter (Prom), type of intron, length of arms and details of how to find the specific sequences of the arms for various gene-silencing constructs are shown. The last four columns show the number of primary independent transformants (or transformed lines where progeny were analysed) showing silencing/the number of transgenic plants produced from the primary transformation experiment.
*Silencing analysis was done on the progeny of the primary transformed plants.

of these genes using the conventional plant technologies of insertional mutagenesis would require vast resources. It has been calculated that to have a 90% chance of finding just one specific single gene (of about 1 kb) in *Arabidopsis* using T-DNA insertional mutagenesis would require the generation of about 350 000 independent transformants (Krysan *et al.*, 1999). The work described in this paper facilitates a directed silencing which, when combined with the efficient, non-tissue culture transformation method for *Arabidopsis* (Clough and Bent, 1998), provides the tools that make the challenge of mirroring in plants, the gene discovery under way in nematodes, more feasible.

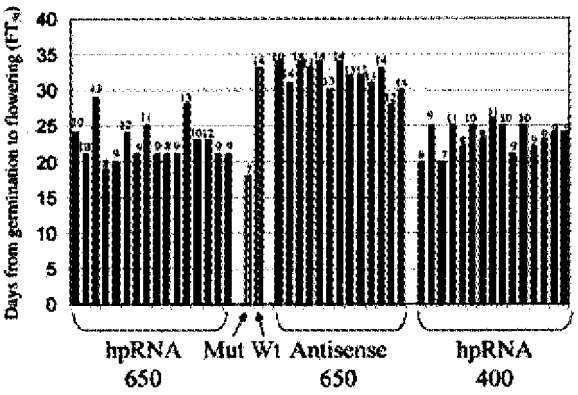


Figure 6. Flowering time in transgenic progeny from independent FLC1-pHANNIBAL and FLC1-anti-sense transformed C24 *Arabidopsis* lines.
Flowering time (FT_{50}) and leaf number (in figures above each column) for transgenic progeny from 16 and 15 independent plant lines transformed with pHANNIBAL constructs containing 650 and 400 nt arms of FLC1 sequence, respectively; 13 independent plants transformed with a conventional, 35S-driven, anti-sense construct containing the appropriate 650 nt of FLC1; the flc-13 mutant (white bar); and wild-type plants (cross-hatched bar). Wild-type plants were transgenic for GUS and kanamycin resistance, to allow the plants to be grown under identical conditions. The standard error for leaf numbers did not exceed 0.7 of 1 day for any plant line.

Using hpRNA constructs, we have obtained silenced plants for every gene that we targeted, irrespective of whether it was a viral gene, transgene or endogenous gene, and the silencing appears to be uniform within tissues in which the hpRNA is expressed. With ihpRNA constructs the efficiency averaged about 90%, and arms of 400–800 nt appear to be stable and effective. High levels of silencing were obtained with constructs having unmatched arm lengths, with arms as long as 853 nt or as little as 98 nt, and with arm sequences derived from coding, 3′ or 5′ untranslated regions of the target gene. These results suggest that ihpRNA constructs will be effective in a wide range of circumstances, and augur well for the generic use of the technology. The silencing was much more profound with ihpRNA constructs than either anti-sense or co-suppression constructs; some ihpRNA transformants were close to exhibiting a complete knock-out of the target endogenous gene. However, most of the ihpRNA plants showed dramatically reduced but detectable levels of target gene activity. This variation in degree of silencing in the ihpRNA plants may be a useful feature for gene discovery and genomics: complete silencing of genes required for basic cell function or development will probably be embryo-lethal and therefore not easily recovered using traditional tagging approaches, whereas the reduced gene expression caused by hpRNA constructs may give viable plants with phenotypes indicative of the role of the target gene.

Although the pHANNIBAL construct should be very useful for studying a modest number of genes (e.g. 10–50), such as in a metabolic pathway, it would not be feasible with normal resources to use it for hundreds to thousands of genes. However, the pHELLSGATE vector has the potential to facilitate making large numbers of gene ihpRNA constructs rapidly and efficiently. The simple steps required, namely PCR, incubation of the PCR product with the vector and recombinase, selection of recombinant plasmid, and then transformation into *Agrobacterium*, are steps that could easily be automated. The templates for PCR could be the defined genes in an EST library using standard forward and reverse primers. Alternatively, given that ihpRNA constructs with arms as small as 98 nt give effective silencing, oligosynthesizers could be automated to systematically synthesize oligonucleotides of each computer-identified gene along a chromosome, or for genes for which no function is known, and pass these primers into an automated ihpRNA production system.

It has been shown that RNAi in *Drosophila* is directed by 21 nt dsRNA oligomers derived from the inducing dsRNA (Elbashir *et al.*, 2001; Zamore *et al.*, 2000). Similar 21–25 nt RNAs have also been found associated with PTGS in plants (Hamilton and Baulcombe, 1999; Waterhouse *et al.*, 2001). It is tempting to speculate that the minimum region of homology between an mRNA and the arms of an effective hpRNA will also be 21–25 nt. If so, this rule would allow the design of hpRNAs to silence a single member of a gene family, as such unique sequences are present in most gene families. Also, by choosing conserved regions, it may be possible to silence whole gene families using a single construct. However, these rules remain to be proven.

Experimental procedures

Plasmid construction

Standard gene cloning methods (Sambrook *et al.*, 1989) were used to make the gene constructs. The plasmids for dicot transformation were derived from pART7 and pART27 (Gleave, 1992), and those for monocot transformation were derived from pVec4 (Wang *et al.*, 1997, Wang *et al.*, 1998). The accession numbers of the gene sequences, and the co-ordinates of the sequences used in the hpRNA, co-suppression and anti-sense constructs, are shown in Table 1. The annotated sequences of pHANNIBAL, pKANNIBAL and pHELLSGATE are lodged with EMBL and have accession numbers AJ311872, AJ311873 and AJ311874, respectively. Constructs made in pHANNIBAL were subcloned as *Not*I fragments into pART27, then introduced into *Agrobacterium* strains AGL1 or LBA4404 either by electroporation or tri-parental mating. pHELLSGATE was maintained in *E. coli* strain DB3.1 (Invitrogen, Carlsbad, CA, USA) in which the *ccdB* gene is not lethal.

Plant transformation

Nicotiana tabaccum (W38), cotton and rice were transformed essentially as described by Ellis *et al.* (1987), Cousins *et al.* (1991) and Wang *et al.* (2001), respectively. *Arabidopsis* was transformed by the dipping method of Clough and Bent (1998).

Analysis of transgenic plants

Northern blot analysis for the presence or absence of short RNAs was performed essentially as described by Wang *et al.* (2001).

Polyphenol oxidase (PPO) activity was measured using an oxygen electrode essentially as described by Robinson and Dry (1992). Rice and tobacco were tested for GUS activity using the histochemical stain X-glucuronide essentially as described by Jefferson *et al.* (1987). The reactions of plants to potato virus Y were analysed as described by Waterhouse *et al.* (1998). The activity of EIN2, which is required in the ethylene perception pathway, was observed by growing plants on media containing 1-aminocyclopropane-1-carboxylic acid (ACC) as described by Alonso *et al.* (1999). To identify lines silenced for EIN1, at least 30 progeny of each transformed line were germinated and grown on ACC-containing media.

To measure the effect of silencing FLC1, 20–30 seeds from each transgenic C24 *Arabidopsis* line, the transposon mutant flc13 (Sheldon *et al.*, 2000), and a control GUS line, were germinated and grown on kanamycin plates as described by Sheldon *et al.* (2000). The plants were scored daily over a 40-day period for the appearance of flowers. Flowering time (FT_{50}) for each line was taken as the number of days after germination for 50% of the plants to show flowering. After flowering, the number of leaves of 10 randomly selected plants was counted for each line.

Chalcone synthase (CHS) activity was monitored by visual observation of stem and leaf colour in plants grown under high light, and by unaided or microscope-assisted visual observation of seed-coat colour. The seeds were collected after they had matured and dried on the plant. The relative flavonoid concentrations in seeds were determined by measuring extracts for absorbance between 490 and 530 nm in a Spectramax 340-PC (Molecular Devices Corporation, Sunnyvale, CA, USA). Duplicate extracts were made from 25 mg seed of each line, essentially as described by Gerats *et al.* (1982). The average absorbance value for each line was mathematically transformed to give relative values such that the *tt4* and wild-type seed became values of 0 and 100%, respectively.

The activity of Δ12- and Δ9-desaturase activity during lipid synthesis was estimated from the relative proportions of individual fatty acids in mature seed, as determined by routine methods for GC analysis of fatty acid methyl esters.

Unless otherwise stated, plants were considered to be showing silencing when they showed obvious appropriate phenotypic differences from wild-type plants, or when they had a gene activity that was reduced by at least 20%.

AttB primers, PCR and recombination reaction for introduction of sequences into pHELLSGATE

Primers with attB1 and attB2 sequences were purchased from Life Technologies. Polymerase chain reactions (PCR) and *in vitro* BP clonase recombination reactions were carried out according to the manufacturer's instructions (Invitrogen). The recombination reaction product was either electroporated or heat-shocked into RbCl-treated DH5α *E. coli*.

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